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This work was undertaken to determine if 6-aminonicotiamide (6AN) can enhance efficacy of radiation (XRT) and/or chemotherapy (paclitaxel or adriamycin). The study was done in hormone resistant (MDA-MB-435) and hormone sensitive (MCF-7) breast tumors. The interval between 6AN and XRT/chemotherapy was determined by 31P NMR spectral changes. Spectral changes were noted post 6AN in perfused MCF-7 and MDA-MB-435 cells including a decrease in the ratio of phosphocreatine to inorganic phosphate (Pi) and an increase in 6-phosphogluconate (a product of the pentose phosphate pathway) to Pi. Similar changes were noted in vivo in the MCF-7. 6AN enhanced the effect of XRT (2 Gy) and adriamycin in the MCF-7 but inhibited the effect of paclitaxel. In the MDA-MB-435, no enhancement was noted in vitro. In vivo, 6AN enhanced the effect of radiation (5Gy/fraction) and adriamycin in the MCF-7. Treatment with paclitaxel was too toxic with 6AN to evaluate. In the MDA-MB-435, enhancement of XRT was noted with 6AN, but not of adriamycin. The latter may have been to a lower dose of adriamycin (5mg/kg) than was used in the MCF-7. 6AN inhibited paclitaxel in the MDA-MB-435. 6AN was effective at non-toxic doses in enhancing the effect of XRT in both MCF-7 and MDA-MB-435, but inhibited paclitaxel in the latter tumors

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Introduction

This report represents the final 3 year summary of the experiments performed under this grant/contract. The report draws heavily (sometimes verbatim) from the reports for years 1 and 2.

The focus of this research was to evaluate the potential of 6-aminonicotinamide (6AN) to enhance response of breast tumors to chemotherapy (adriamycin and paclitaxel) and/or radiation (XRT). The aims of the overall project are to test 6AN, both in vivo and in vitro, on two tumor models, a hormone resistant and hormone sensitive model (MCF-7).

Body

Cell Studies

Methods: *Cell culture.* MCF-7 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Essential Medium (DME) and Minimal Essential Medium (MEM) mixed 1:1 and supplemented with 5% fetal bovine serum, 100U/ml penicillin-G and 100ug/ml Streptomycin, 1mM pyruvate and 0.25 U/ml bovine insulin (Intergen, Purchase, NY). The concentration of glucose in the medium was 15 mM. MEM was supplemented with non-essential amino acids and the final concentration of each was 0.5mM. For NMR studies, cells were grown on Cultispher gelatin beads (HyClone Laboratories, Inc., Logan, UT) in the same medium described above except that it was supplemented with 10% FBS. Cells ($1 - 2 \times 10^7$) were mixed with 0.2 g beads in 50ml medium inside a spinner flask. Cells and beads were left 18 - 24 hours without stirring. Subsequently, cells and beads were stirred at 30 - 40 rpm. Cells were cultured on the beads for 5 - 7days before being used for NMR studies. Readiness of cells was judged visually by mixing a small amount of cells with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma) which is taken up by cells and converted to an insoluble, purple product by dehydrogenases. After 30 minutes, beads were inspected under a microscope to see the extent of cell confluence on the beads. Experiments were started if greater than 90% of the beads were greater than 50% covered with cells.

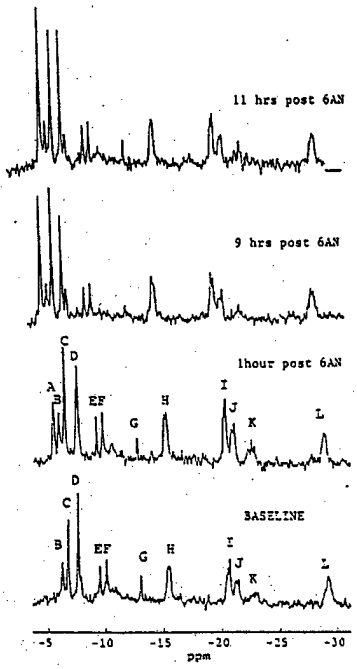
For cytotoxicity studies, 1.3×10^5 cells were subcultured in T-25 flasks. Four days later cells were exposed to 6-AN for 4 hours. Media containing 6-AN was replaced with fresh media and 3 hours later cells were exposed to radiation, adriamycin or paclitaxel. In the case of adriamycin, cells were exposed for 4 hours; in the case of paclitaxel cells were exposed for 24 hours. At the end of adriamycin and paclitaxel exposures, cells were trypsinized and counted using a Coulter Counter. After radiation, cells were returned to the incubator for 3 hours of recovery and then trypsinized and counted. Cells (400 - 2000 cells) were plated into 6-well plates containing 2 ml media described above but supplemented with 5 or 10% FBS and 1U/ml insulin. After 2 - 3 weeks, clones of over 100 cells were counted by staining with 1% crystal violet in absolute methanol.

NMR measurements. MCF-7 cells grown on collagen beads were placed into a 10mm (or 12mm) shortened, screw-cap NMR tube which was connected to a perfusate reservoir via tubing inserted through a teflon/silicone septum (1,2). Inlet tubing carried perfusate from the 500 ml reservoir-bottle to the bottom of the NMR tube. The pumping speed was adjusted so that the beads floated to the beginning of the widest cross-sectional area of the tube, about a centimeter from the top. Outlet tubing carried perfusate from the top of the NMR tube, above the top of the beads, back to the reservoir bottle. The outlet tubing ended with a T-connection that allowed switching between the perfusate-reservoir (closed system) and a waste-reservoir (open system). Switching to an open system allowed washout of drug. The perfusate consisted of phosphate-free DME supplemented with 10% FBS, 1mM pyruvate and 0.25U/ml insulin. Oxygenation of the perfusion media was accomplished by blowing O_2/CO_2 (95%:5%) over the top of the media in the reservoir.

NMR spectra of phosphorus metabolites were collected using a five-turn solenoid coil that was wrapped around the NMR tube (1,2). WALTZ decoupled ^{31}P spectra were obtained using a

Bruker/GE 4.7 T Omega spectrometer (Bruker NMR, Fremont, CA) operating at 81.03 MHz. Data was acquired with a spectral width of 10,000 Hz, a 60° pulse angle, a recycle time of 2 seconds, 2048k data points and 2048 signal averaged free-induction decays (FID). To quantify metabolites, FID's of 2 sequential acquisitions were summed to improve signal-to-noise and the areas under the curve for metabolites of interest were estimated using Magnetic Resonance User Interface software package (MRUI). The AMARES fitting routine was employed to fit a Lorentzian function to the time domain signal. Spectra were processed with 5 Hz exponential line broadening.

Methodology for the in vivo 31P NMR spectra and tumor growth delay studies are similar to our previous studies (3,4) with the addition that the MCF-7 tumors require implantation of a slow release estrogen pellet. Tumor were inoculated on the flank of nude mice and were studied when they attained a volume of ~ 150 mm3. In the early studies mice were injected when they were approximately 10 weeks old, although in later studies, younger mice (6-8 weeks old) were used. The tumors grow more rapidly in the younger mice and we have modified our technique to purchase mice when they are 6 weeks old and inject them with tumor cells within 1-2 weeks after delivery. Our experimental methods included running cohorts of controls (no treatment) on each experiment to allow for variations such as these. To evaluate tumor response, tumor regrowth after treatment was measured. In experiments involving radiation (XRT), we chose to measure the time it took for the tumor to regrow to its initial tumor volume. Data are reported as mean +/- standard error of the mean (SEM). Tumor volume was measured as the product of (d1 xd2 x d3)π/6 where d1, d2, and d3 are three perpendicular diameters. Mice were treated on day 0, day 11 and day 21 unless otherwise stated (paclitaxel study was the only deviation from this schedule because of toxicity). The interval between 6AN and adriamycin or XRT was 10 hours. The dose of 6AN for all experiments was 16 mg/kg based on work accomplished in year 1 of the study. This was noted to be decreased compared to the CD8F1 mice which tolerated 20 mg/kg. Mice who died within the first 28 days of the study were censored from the data analysis and were considered treatment related deaths and is reported below.



Results

Tasks 1,3. NMR Perfused Cell Studies: The perfusion scheme for MCF-7 cells was validated. The perfused cells (MCF-7) were metabolically stable for 48 hours when perfused with phosphate-free DME (data not shown). A higher signal-to-noise ratio was achieved with cells grown on beads using the petri dish method than with cells grown on beads in a spinner flask. This was probably due to greater cell density as indicated by the qualitative observation that beads incubated with cells in a petri dish had greater MTT

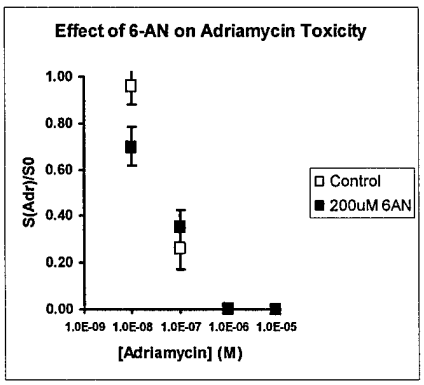


Figure 3. Effect of 6AN on efficacy of Adriamycin. Note decrease in surviving fraction at 10^{-8} dose.

staining than beads incubated with cells in a spinner flask. NMR coils have been constructed both for in vivo and in vitro studies.

The effect of 6AN on tumor metabolism has been studied (MCF-7). Spectra have been obtained both without (data not shown) and with 6AN (Figure 1, reference 5, and Appendix 1). 6-phosphogluconate (6-PG) levels rose from undetectable in the baseline spectrum to become the largest peak in the spectrum. Quantitative assessment of the effect of 6-AN on 6-PG/Pi, and PCr/Pi is demonstrated in Figure 2. 6-PG/Pi rose to a maximum level by 10 hours after the end of the 6-AN perfusion. There was no change in βNTP levels but PCr levels decreased indicating that energy metabolism was inhibited. (see Fig. 1). Peaks detected include A= 6-phosphogluconate (6PG), B= phosphoethanolamine (PE), C=phosphocholine (PC), D=inorganic phosphate (Pi), E=glycerophosphoethanolamine (GPE), F=glycerophosphocholine (GPC) , G= phosphocreatine(PCr), H, I and L= γ, α, and β nucleoside triphosphate, J = NAD(H) and K=diphosphodiesters

Task 4. A 4 hour exposure to 6-AN (200um) decreased plating efficiency by 10% (Table 1). At 2 Gy, a modest effect was noted by combining 6AN and 2 Gy but the effect was lost at higher doses (4 Gy and 6 Gy). There was no effect on surviving fraction by adding 6AN to paclitaxel..

Task 4. 6-AN increased the effectiveness of 10^{-8} M adriamycin to decrease MCF-7 cell clonogenicity ($p<0.05$) but had no effect when adriamycin was present at higher levels. This finding may indicate that the two drugs have in common cellular processes or targets which are damaged or changed and through which the drugs exert their cytotoxic effects. At low levels of adriamycin these process or targets are not completely saturated and thus the addition of 6-AN may cause an increase in cytotoxicity. At higher levels of adriamycin, there is significant cell kill and therefore and the apparent potentiation by 6-AN disappears. (Fig 3)

NMR Cell Studies-MDA-MB-435

Tasks 2, 3. We had difficulty with growing the MDA-MB-231 (slow growth rate) so the MDA-MB-435, which is also hormone resistant, was selected for study. The MDA-MB-435 grew to 150 mm3 in 3-4 weeks and studies with this tumor model were therefore initiated.

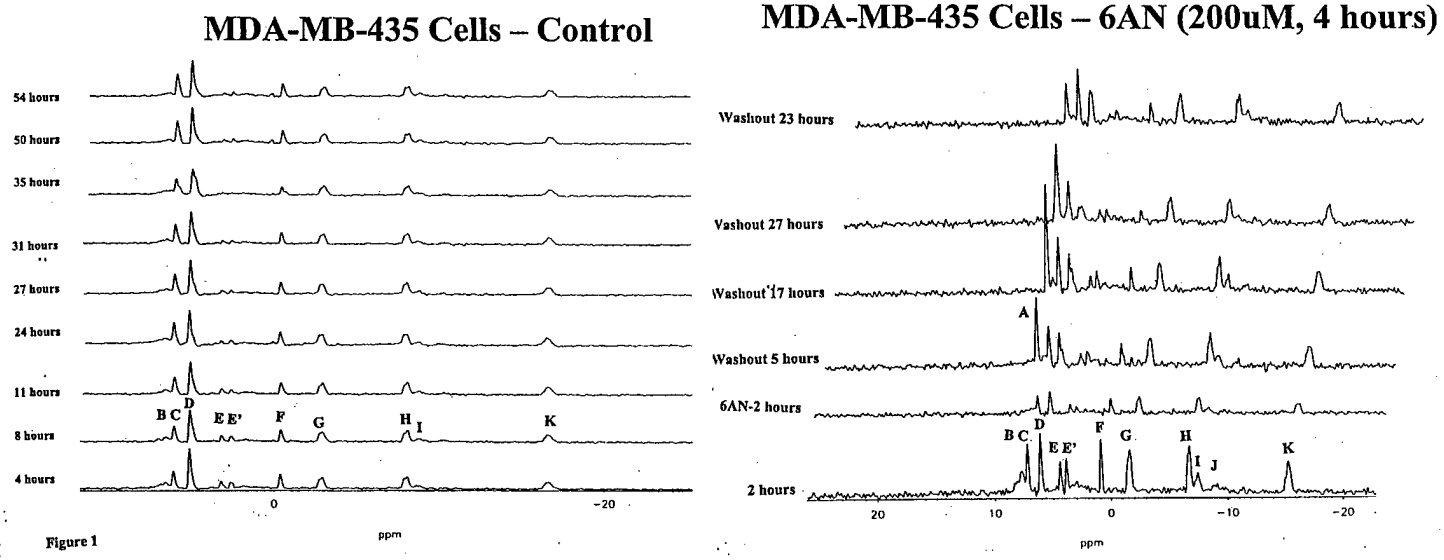


Fig. 4 31P NMR spectra of MDA-MB-435 cells with and without treatment with 6AN. Note stability of spectra on the left compared to the presence of a new peak (peak A= 6PG) and loss of PCr posttreatment with 6AN (right panel)

Figure 4 (left) shows a control 31P NMR study demonstrating that the perfused MDA-MB-435 cells are stable for periods of >50 hours while being perfused in the NMR system. The spectral peaks are identified as B= phosphoethanolamine, C=phosphocholine, D=inorganic phosphate (Pi), E=glycerophosphoethanolamine, E'=glycerophosphocholine, F= phosphocreatine, G, H and K= γ, α, and β nucleoside triphosphate, I = NAD(H) and J=diphosphodiesters. Note that the spectra are stable over the duration of the study.

In contrast, figure 4 (right) shows MDA-MB-435 cells treated with 6AN (200uM). The initial (pretreatment) spectra are similar to data shown in figure 1. However, treatment with 6AN leads to a loss of PCr and NTP, an increase in Pi and splitting of the Pi peak into different components, indicating a region of pH heterogeneity. These findings are similar to our data with the MCF-7. Surviving fraction experiments to measure the effect of 6AN on cellular radiosensitivity and enhancement of the effect of paclitaxel were done (Table 2).

TASK 4 Table 2 shows that 6AN failed to enhance the effect of radiation. Although the surviving fraction decreased when 6AN was combined with 2 Gy, 6AN alone had a comparable effect and thus no gain could be ascribed to the combination. The results with paclitaxel were similar – some modest benefit was seen at 10^{-8}M but the effect was lost at 10^{-7}M.

In Vivo 31P NMR Studies (MCF-7)

Task 5 31P NMR spectra from MCF-7 tumors treated with 6AN were obtained using previously described techniques and are presented in Figure 5. The expected peaks including phosphoethanolamine (PE), phosphocholine (PC), inorganic phosphate (Pi), phosphodiester (PDE), phosphocreatine (PCr), γ, α, and β nucleoside triphosphates (NTP), NAD(H). Post treatment with 6AN, the same spectral peaks are visible but an additional peak is seen in the most downfield (left) portion of the spectrum. This peak has been previously assigned to 6-

phosphogluconate (6PG). Based on the lack of significant change in the high energy phosphates over time (Figure 6), it was decided to use a 10 hour interval between 6AN and radiation or chemotherapy based on changes in 6PG.

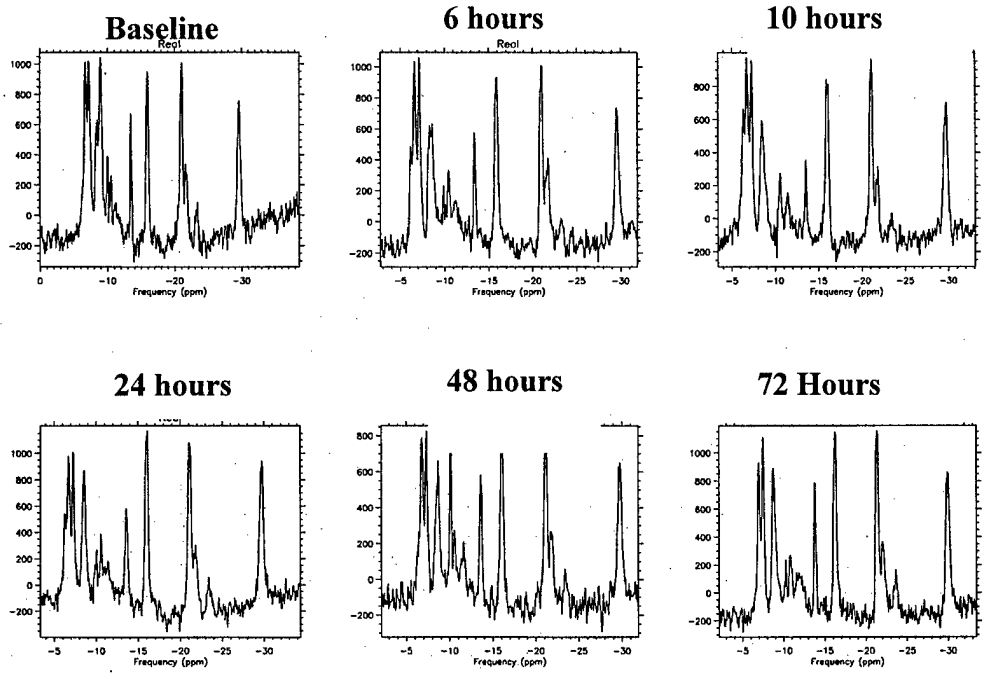


Figure 5. Change in 31P NMR spectrum post 6AN. Note appearance of new peak on the left (downfield) at 6, 10 and 24 hours post 6AN

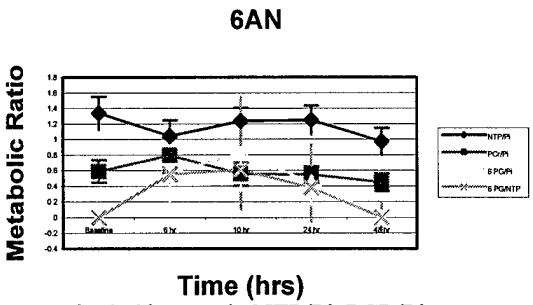


Fig.6 Changes in NTP/Pi, PCR/Pi, 6PG/Pi and 6PG/NTP after treatment of MCF-7 tumors in vivo. Changes in PCR/Pi and NTP/Pi were not significant.

Tumor Growth Delay Studies (MCF-7)

Tasks 5 (continued), 6, 7. Tumor doubling time for the MCF-7 (n=25) was 22.0 +/- 2.1 days (Mean +/- SEM). We initially examined the effect of 6AN on radiation sensitivity. Tumor growth delay was assayed based on the time it took for the tumor to regrow to its pretreatment value. In almost all cases, repeat controls (6AN alone and control were done), thereby explaining the relatively large n value for the 6AN cohort. None of the untreated tumors experienced tumor shrinkage and therefore no regrowth delay is reported. Table 3 summarizes the in vivo data with the MCF-7 (hormone sensitive tumor). The major finding was that 6AN

clearly enhanced response to XRT. The regrowth delay for 5 Gy administered on days 1, 10/11 and 21 was 22.6 +/- 3.1 days. In contrast, the regrowth delay for tumors treated with 6AN→ 5 Gy was >52.3 +/- 4.1 (not all tumors had regrown to their initial tumor volumes when the experiment was terminated). The difference between XRT vs. 6AN→ XRT was significant ($p<0.01$). To evaluate the effect of 6AN on radiation enhancement, we measured the regrowth delay for mice receiving 10 Gy on the identical schedule which was 49.4 +/- 3.8 days. The regrowth delay for 5 vs. 10 Gy was significant ($p<0.05$). The regrowth delay for 10 Gy vs. 6AN→5Gy was not significant indicating that 6AN has a dose modification factor as a radiation enhancing agent of 2, i.e. half the dose of radiation is equally effective when combined with 6AN.

We also examined the effect of 6AN on response to adriamycin. In previous experiments with the CD8F1 tumor, the maximum tolerated dose (MTD) of adriamycin was 11 mg/kg on this schedule (3 doses over 3 weeks). However, nude mice are more fragile and the MTD was only 10 mg/kg (28 day mortality was 2/15 mice) in our initial study. It is noted that the eventual 28 day mortality rate was 4/25 (16%) which is excessive, suggesting that a dose of 9 or 9.5 mg/kg would have been more appropriate (although probably less efficacious). The 28 day mortality of mice treated with 11mg/kg was 3/9. Tumor regrowth delay for mice treated with adriamycin (10mg/kg) was 11.4 +/- 4.4 vs. 25.8 +/- 6.7 for mice receiving 6AN→adriamycin (6mg/kg) which was significant ($p=0.05$). A relatively steep dose response curve was noted for Adriamycin since if one lowered the dose to 5mg/kg, the enhancement was lost (with approximately equal mortality).

In comparison, there was no enhancement with 6AN→paclitaxel. The dose of paclitaxel used was 12mg/kg q12 x 3 doses for each time point (days 1, 10/11 and 21) which we have used previously without significant mortality (6). With nude mice, the mortality rate was 20%. When combined with 6AN, it was only possible to give 2 doses (second dose reduced to 10 mg/kg x 3) which still had a 37% mortality rate. In view of the enhanced toxicity, response could not be evaluated although there was a suggestion of enhanced effect.

In the MCF-7 tumor, tumor growth delay measurements and evaluation of tumor response indicate that 6AN is effective at enhancing response to radiation (5 Gy). There is also an enhanced response as measured by tumor growth delay for mice receiving 6AN→adriamycin compared to mice treated with Adriamycin alone.

Tumor Growth Delay Studies (MDA-435)

Tasks 8 and 9. Tumor growth delay studies were done with the MDA-435 with the same techniques as noted for the MCF7. These results are summarized in Table 4

None of the untreated tumors experienced tumor shrinkage and therefore no regrowth delay is reported. The doubling time for the MDA-MB-435 (n=15) was 20.4 +/- 3.2 days (Mean +/- SEM). Table 2 summarizes the in vivo data with the MCF-7 (hormone sensitive tumor). It is noted that 6AN had modest anti-tumor effect (regrowth delay=13.4 +/- 3.1). The major finding was that 6AN clearly enhanced response to XRT in the MDA-435. The regrowth delay for 5 Gy

administered on days 1, 10/11 and 21 was 21.2 +/- 6.5, which was very similar to that observed for the MCF-7. In contrast, the regrowth delay for tumors treated with 6AN→ 5 Gy was >53.7 +/- 13.4 (not all tumors had regrown to their initial tumor volumes when the experiment was terminated). The difference between XRT and 6AN→ XRT was significant ($p<0.05$). To evaluate the effect of 6AN on radiation enhancement, we measured the regrowth delay for mice receiving 10 Gy on the identical schedule which was 54.5 +/- 11.8 days. The regrowth delay for 5 vs. 10 Gy was significant ($p<0.05$). The regrowth delay for 10 Gy vs. 6AN→5Gy was not significant indicating that 6AN has a dose modification factor as a radiation enhancing agent of 2, i.e. half the dose of radiation is equally effective when combined with 6AN.

We also examined the effect of 6AN on response to adriamycin. The 28 day mortality rate was 1/10 (10%) indicating that 10mg/kg was an appropriate MTD. Tumor regrowth delay for mice treated with adriamycin (10mg/kg) was 16.5 +/- 5.2 vs. 25.8 +/- 6.7 for mice receiving 6AN→adriamycin (5mg/kg) which was not significant. Since the mortality rate was 10% for the latter cohort, no attempt was made to increase the dose to 6mg/kg.

There was no enhancement with 6AN→paclitaxel. Based on our previous studies with the MCF-7 tumor, the dose of paclitaxel was lowered to 10mg/kg which resulted in an acceptable 10% mortality rate and a regrowth delay of >46.4 +/- 4.9. When combined with 6AN, a decreased tumor regrowth time was clearly evident (26.1 +/- 2.8) which was significant. Comparable mortality (10%) between the two cohorts was noted.

Table 1. Summary of Clonogenic Survival of MCF-7 Mammary carcinoma *(in vitro)* with single and combined treatments.

Treatment	n	n (evaluable)	Mean #colonies/dish	Plating efficiency (%)	Surviving fraction (clonogens)*	
MCF-7						
Controls	14	14	113.3	57.0	1.0	
2Gy	9	9	90.0		0.8	
4Gy	9	9	84.0		0.15	
6Gy	8	8	170.0		0.03	
6AN (200um)	6	6	102.0	51.0	0.90	
TAXOL						
(10^{-8}um)	3	3	99.3		0.75	
(10^{-7}um)	6	6	78.0		0.69	
6AN + TAXOL	13	13	171.3		0.75	
2Gy + 6AN	3	3	61.3		0.54	
4Gy + 6AN	3	3	62.3		0.11	
6Gy + 6AN	6	6	146.8		0.03	
2Gy + TAXOL	3	3	45.7		0.40	
4Gy + TAXOL	6	6	78.3		0.14	
6Gy + TAXOL	3	3	72.7		0.01	
2Gy + 6AN + TAXOL	3	3	127.3		0.25	
4Gy + 6AN + TAXOL	12	12	51.7		0.09	
6Gy + 6AN + TAXOL	3	3	88.0		0.02	

Table 2. Summary of Clonogenic Survival of MDA435 Mammary carcinoma *(in vitro)* with single and combined treatments.

Treatment	n	n (evaluable)	Mean #colonies/dish	Plating efficiency (%)	Surviving fraction (clonogens)*	
MDA435						
Controls	15	12	153.3	77.0	**1.0**	
2Gy	9	6	162.0		**0.99**	
4Gy	12	10	65.4		**0.17**	
6Gy	9	9	45.0		**0.03**	
6AN (200um)	14	12	80.0	80.0	**0.52**	
TAXOL						
(10^{-8}um)	9	6	126		**0.83**	
(10^{-7}um)	9	9	116		**0.55**	
6AN + TAXOL	18	12	86.6		**0.28**	
2Gy + 6AN	9	9	91		**0.59**	
4Gy + 6AN	9	9	26.4		**0.07**	
6Gy + 6AN	9	9	40.5		**0.03**	
2Gy + TAXOL	6	6	130.3		**0.17**	
4Gy + TAXOL	9	9	46.2		**0.10**	
6Gy + TAXOL	9	9	42.0		**0.01**	
2Gy + 6AN + TAXOL	12	9	240.7		**0.39**	
4Gy + 6AN + TAXOL	9	9	33.2		**0.04**	
6Gy + 6AN + TAXOL	9	6	52.8		**.026**	

n = number of tissue culture dishes plated

* **Surviving fraction** = $\frac{\text{surviving colonies}}{\text{PE x (cells plated)}}$

Table 3. Tumor Growth Delay – MCF7

Cohort Treatment	n	28day mortality	Regrowth Time (days)Mean +/- SEM
6AN	35	2/37	8.7 +/- 1.8
5 Gy	27	0/27	22.6 +/- 3.1
10 Gy	16	0/16	49.4 +/- 3.8
6AN + 5Gy	25	2/27	>52.3 +/- 4.1
Adriamycin (10mg/kg)	21	4/25	11.4 +/- 4.4
6AN→Adriamycin (6mg/kg)	14	1/15	25.8 +/- 6.7
6AN→Adriamycin (5mg/kg)	13	2/15	13.4 +/- 4.7
Paclitaxel 12mg/kg q12h x 3 doses	8	2/10	79.8 +/- 6.9
6AN (16mg/kg; days 1 and 11)→ Paclitaxel (12mg/kg day 1 and 10mg/kg day 11)	5	3/8	Not evaluated due to toxicity

Table 4. Tumor Regrowth Times - MDA-MB-435

Cohort Treatment	n	28 day mortality	Regrowth Time (days)
Control	15		0
6AN	19	0	13.4 +/- 3.1
5 Gy	8	0	21.2 +/- 6.5
10 Gy	8	0	54.5 +/- 11.8
6AN+5Gy	10	0	>53.7 +/- 13.4*
Adria (10mg/kg)	10	0	16.5 +/- 5.2
6AN+Adria (5mg/kg)	9	1/10	11.8 +/- 2.5
Taxol (10mg/kg x 3, IV)	9	1/10	>46.4 +/- 4.9
6AN + Taxol	9	1/10	26.1 +/- 2.8

Key Research Accomplishments

1. 6AN has been shown to enhance the effect of XRT (5 Gy fractions) in two tumor models. The effect of 6AN + 5Gy was equivalent to 10 Gy alone (in both tumor models), indicating a significant biological effect which may be of clinical relevance.
2. 6AN enhances efficacy of adriamycin when the latter is administered at 6 mg/kg but not at 5 mg/kg. This may be clinically significant since support of patients maybe more feasible than mice.
3. 6AN inhibited the effect of paclitaxel in vivo.

Reportable Outcomes

1. Holleran A, Chen, Y, and Koutcher JA. Effect of 6-aminonicotinamide on human tumor metabolism and response to chemotherapy and radiation. Int. Soc. Magn Reson in Med. 7th Ann Mtg. Phil. Pa. May 23-29th, 1999

2 additional manuscript in preparation.

Conclusions

1. Metabolic effects were noted in vitro in both MCF-7 and MDA-MB-435 cells. However, enhancement of cell kill as measured by surviving fraction (beyond additivity) was modest.

The MCF-7 did not exhibit a changes in energy metabolism after 6AN but did exhibit an increase in 6PG. This was used to determine the interval between 6AN and XRT or chemotherapy agents.

In the MCF-7, 6AN enhanced the effect of both XRT (5 Gy fractions) and adriamycin. Paclitaxel was toxic and could not be evaluated.

In the MDA-435, enhancement was noted after 6AN with XRT and adriamycin at 6mg/kg but not at 5mg/kg. The MTD for adriamycin (with 6AN) is between 5 and 6 mg/kg. 6AN inhibited the response of the tumor to paclitaxel resulting in a decreased regrowth time.

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Effect of 6-Aminonicotinamide on Human Tumor Metabolism, and Response to Chemotherapy and Radiation

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Introduction

Previous studies from different investigators (1,2) have indicated that 6-aminonicotinamide (6AN) can enhance anti-neoplastic treatment. In a previous study of RIF-1 cells, the surviving fraction after radiation was decreased if the cells were pretreated with 6AN (1). We have investigated the effect of 6AN on survival of human mammary tumor cells (MCF-7) after treatment with radiation, adriamycin and paclitaxel.

Methods

Cells -- NMR – MCF-7 cells were grown on Culti-spher gelatin beads in media consisting of 50% DMEM and 50% minimal essential media with 10% FBS. Cells (1-2 $x10^7$) were mixed with 0.2 g beads in 50 ml media in a spinner flask for 18-24 hours without stirring. They were subsequently stirred for about 4-6 days at 30-40 rpm. Experiments were started when greater than 90% of the beads were greater than 50% covered with cells.

Cells – cytotoxicity studies – 1.3 x 10^5 cells were subcultured for 4 days and subsequently exposed to 6AN for 4 hours. 6AN containing media was replaced with fresh media and 3 hours later the cells were exposed to radiation (2,3,or 4 Gy), adriamycin (4 hours) or paclitaxel (24 hours). Subsequently, the cells were trypsinized and counted using a Coulter Counter. After radiation, cells were returned to the incubator for 3 hours of recovery and then trypsinized and counted.

NMR – MCF-7 cells grown on collagen beads were placed in a 12 mm shortened screw cap NMR tube which was connected to a perfusate reservoir via tubing. The pumping speed was adjusted so that the beads floated to the beginning of the widest cross-sectional area of the tube, about a cm from the top. NMR spectra were collected using a 5 turn solenoid coil wrapped around the 12 mm NMR tube. WALTZ decoupled 31P spectra were obtained using a 4.7T Bruker Omega CSI system operating at 81.03 MHz. Spectra were obtained prior to perfusion with 6AN, during the 4 hours of 6AN exposure (200uM) and subsequently after washout of 6AN for about 24 hours. Each spectrum required about 70 minutes to acquire.

Results

Figure 1 shows a series of spectra obtained on perfused MCF-7 cells. The peaks detected prior to 6AN perfusion include phosphoethanolamine (PE) (B), phosphocholine (PC) (C), inorganic phosphate (Pi) (D), glycerophosphoethanolamine (GPC) (E), glycerophosphocholine (GPC) (F), phosphocreatine (PCr) (G), γ, α and β NTP (peaks H,I and L), and diphosphodiesters (DPDE) (peaks J and K). The Pi peak was usually split into two components after treatment with 6AN which likely represented resolution of intra- and extra-cellular pH. During the infusion of 6AN and subsequently, a peak previously assigned to 6-phosphogluconate (6PG) was detected (A). As in studies with the RIF-1, this was the dominant peak in the spectrum and was visible for >24 hours post washout of 6AN. The baseline spectrum in Fig. 1 requried 70 minutes while subsequent spectra required 135 minutes of data acquisition.

The effect of 4 Gy of radiation was significantly enhanced by pretreatment with 6AN. The surviving fraction (SF) after 4 Gy was 0.087 ± 0.007 compared to 0.036 ± 0.018 for cells exposed to 6AN before radiation. 6AN alone had no effect on SF; similarly radiation followed by 6AN did not show an enhanced effect.

Pretreatment with 6AN (200uM) resulted in an enhanced efficacy of adriamycin at low concentrations (10^{-8} M), but this effect was lost at higher concentrations. This may be due to the very low cell survival that was present at higher doses of adriamycin. Pretreatment with 6AN followed by paclitaxel failed to show any enhancement. The activity of paclitaxel is dependent on cells traversing mitosis and therefore further studies to determine if 6AN may inhibit cells from entering mitosis are ongoing.

The current study corroborates a previous study (1) which showed enhancement of the effect of radiation by 6AN. The previous study was done in a murine tumor cell line, whereas this study demonstrates radiation enhancement in a human tumor cell line. Furthermore, this study also shows enhancement with adriamycin, one of the two most active agents used in breast cancer. Since adriamycin has a risk of cardiac toxicity at higher doses, it may be feasible by adding 6AN to achieve equi-efficacious activity with a lower dose of adriamycin, thereby decreasing the risk of cardiac toxicity.

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